Active site residues in m-calpain: identification by site-directed mutagenesis

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Abstract Site-directed mutagenesis was used to alter putative active site residues in the large subunit of calpain, and the activity of the mutants was measured following coexpression in *E. coli* of both calpain subunits and purification of the resultant dimers. Mutants Cys105Ser, His262Ala and Asn286Ala had no activity. Together with sequence comparisons among cysteine proteinases, the results suggest that these residues constitute the catalytic triad in calpain. Mutants Asn286Asp and Trp288Tyr had low activity, consistent with interaction of these residues with His262.

Key words: Calpain; Ca^{2+}-binding; Catalytic triad; Cysteine proteinase; Mutagenesis

1. Introduction

The calpains (EC 3.4.22.17) are cytoplasmic Ca^{2+}-dependent cysteine proteinases, found in most cell types of vertebrates and also in some other species (*Drosophila* and *Schistosoma mansoni* [1–3]. Two forms, μ- and m-calpain (calpain I and calpain II), have been well characterized from mammalian tissue. They have similar substrate specificities, but differ in their Ca^{2+}-requirements when assayed in vitro, μ-calpain requiring ~50 μM Ca^{2+} for half-maximal activity and m-calpain 400 μM Ca^{2+}. Both enzymes consist of a catalytic subunit (80 kDa) and a regulatory subunit (30 kDa). The large subunits of μ- and m-calpain are genetically distinct in a given species, while the small subunits are identical. The 3D structure of calpain has not yet been solved, but on the basis of amino acid sequence it appears that the large subunits consist of four domains [4]. Domain II contains the active site residues, while domain IV of the large subunit (as well as the C-terminal domain of the small subunit) contains several EF hand sequences which may bind Ca^{2+}.

All cysteine proteinases so far characterized involve a pair of cysteine-histidine residues at the active site, referred to as the catalytic S^-/ImH^+ ion pair [5,6]. Very little biochemical information is available about the active site of calpain, but the active site cysteine residue was identified as C105 by means of labelling with [^{14}C]iodoacetic acid [7], and this has been confirmed recently by mutagenesis [8]. Sequence comparisons among cysteine proteinases have suggested that H262 in calpain is likely to be the second component of the catalytic ion pair, and that N286 could be involved in stabilising this ion pair [4,5,9]. There are several other residues near the active site which are highly conserved throughout the different families of cysteine proteinases, but the reasons for their strict conservation are in many cases not yet clear.

The calpains form a distinct family within the general class of cysteine proteinases. Although domain II of calpain has sometimes been described as papain-like, the percent amino acid sequence identity with papain and with other cysteine proteinases is low, and is insufficient to provide structural information. A further difference is that the pK_a values of the presumed catalytic ion pair in calpain are different from those of other cysteine proteinases [10], suggesting differences in the active site regions. For these reasons, predictions about calpain activity based on papain or similar proteinases must be made with care.

There are other major distinctions which make the calpains worthy of further study. Most obviously, the regulation of calpain by Ca^{2+} represents a level of control not seen in the other cysteine proteinases. This regulation is generally assumed to involve a conformational change in calpain induced by Ca^{2+}-binding to the C-terminal domains of both subunits, although the mechanism whereby this change affects the active site residues in domain II remains to be established.

In addition to these considerations, the possible involvement of calpain in several disease states [11] makes it a potential drug target, and a precise description of the active site region would help in drug design. For calpain, the recent development of a satisfactory bacterial expression system [8,12] has made possible a study of active site residues by means of site-directed mutagenesis, and we report here some results of this approach applied to recombinant rat m-calpain.

2. Materials and methods

2.1. Materials

Native m-calpain isolated from mammalian tissues consists of an 80+30-kDa dimer, the smaller subunit of which is very rapidly N-terminally truncated by autolysis when the enzyme is activated [13]. The rat m-calpain defined in this paper as wild-type was obtained by coexpression in *E. coli* of an intact large subunit (80 kDa) with a truncated form (21 kDa) of the small subunit which corresponds closely to the natural autolysis product [8]. The numbering of residues in the calpain large subunit is that of human and rat m-calpain [14].

2.2. Mutagenesis

Mutants of the large subunit were made in the wild-type clone in pET-24d(+) by site-directed mutagenesis. Antisense primers with the sequences: 5′-ccagaagccagctgtccccaagggc-3′(C105S); 5′-gacggagtacgaggatcctttcaccaact-3′(H262A); 5′-tgtccccaggggccctgatacgtatc-3′(N286A); 5′-gtccccaggggtcgcgaatacgtatcaa-3′(N286D); and 5′-ctccacttgtccatatgggttcctgatac-3′(W288Y) were used, and in each case gave rise to a diagnostic restriction site (underlined). The sequences around the mutation sites were confirmed by DNA sequencing.

2.3. Expression and purification

The calpain large subunit mutants and a 21-kDa fragment of the rat

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small subunit were coproduced from compatible plasmids in *E. coli* BL21(DE3), and the resulting calpains were purified by chromatography on DEAE-Sepharose and Spectra/Gel AcA 44 as previously described [8,12]. A final step of purification was carried out on an FPLC Q-Sepharose column. All buffers used contained 5 mM EDTA, 10 mM β-mercaptoethanol and 0.2 mg/ml PMSF to minimise oxidation or breakdown of calpain during purification.

Gel electrophoresis, and determination of calpain activities and protein concentrations, were carried out as previously described [8,12].

2.4. Light scattering

Light scattering was measured using a Perkin Elmer LS50 fluorometer equipped with a magnetic stirrer under the cuvette holder. Emission and excitation wavelengths were both set at 340 nm, with slit widths of 10 nm, and the light source was attenuated using the machine filter. Calpain samples (75 μg/ml, 0.75 μM) were briskly stirred at room temperature in 5 mM Tris-HCl, 1 mM EDTA, pH 8.0; after observing base-line values, the rate of increase of light scattering was measured for several minutes following addition of $CaCl_2$ to a net final concentration of 2 mM. The aggregation was reversible on addition of excess EDTA.

3. Results

All five calpain mutants described here (C105S, H262A, N286A, N286D and W288Y) were produced as hetero-dimers at protein levels similar to those obtained with the active wild-type enzyme, and at least 70% of the expressed protein was recovered in the soluble fraction after sonication of the cells. During purification of the mutants, the major calpain-containing peaks eluted in the positions expected for wild-type calpain. The cell lysates for two of the mutants (H262A and N286A) contained a slightly higher proportion of aggregated or partially unfolded calpain which was separated out during purification. The purity of the mutants after the FPLC Q-Sepharose column is shown in Fig. 1.

On measurement of Ca^{2+}-dependent proteinase activity, with casein as substrate, the W288Y mutant was found to be the most active of the mutants, with a specific activity of 128 U/mg (Table 1). This value is ~5% of that of the wild-type enzyme, which is 2300 U/mg [8]. The shape of the Ca^{2+}-titration curve for the W288Y mutant, but not the [Ca^{2+}] required for 50% of maximum activity, was significantly altered from that of the wild-type enzyme (Fig. 2). The N286D mutant showed a very slight activity of 22 U/mg, and the C105S, H262A and N286A mutant dimers showed no activity, even when 60 μg of calpain were included in the assay. The active site concentrations of these mutants could not be determined by inhibitor titration due to their low or non-existent activities.

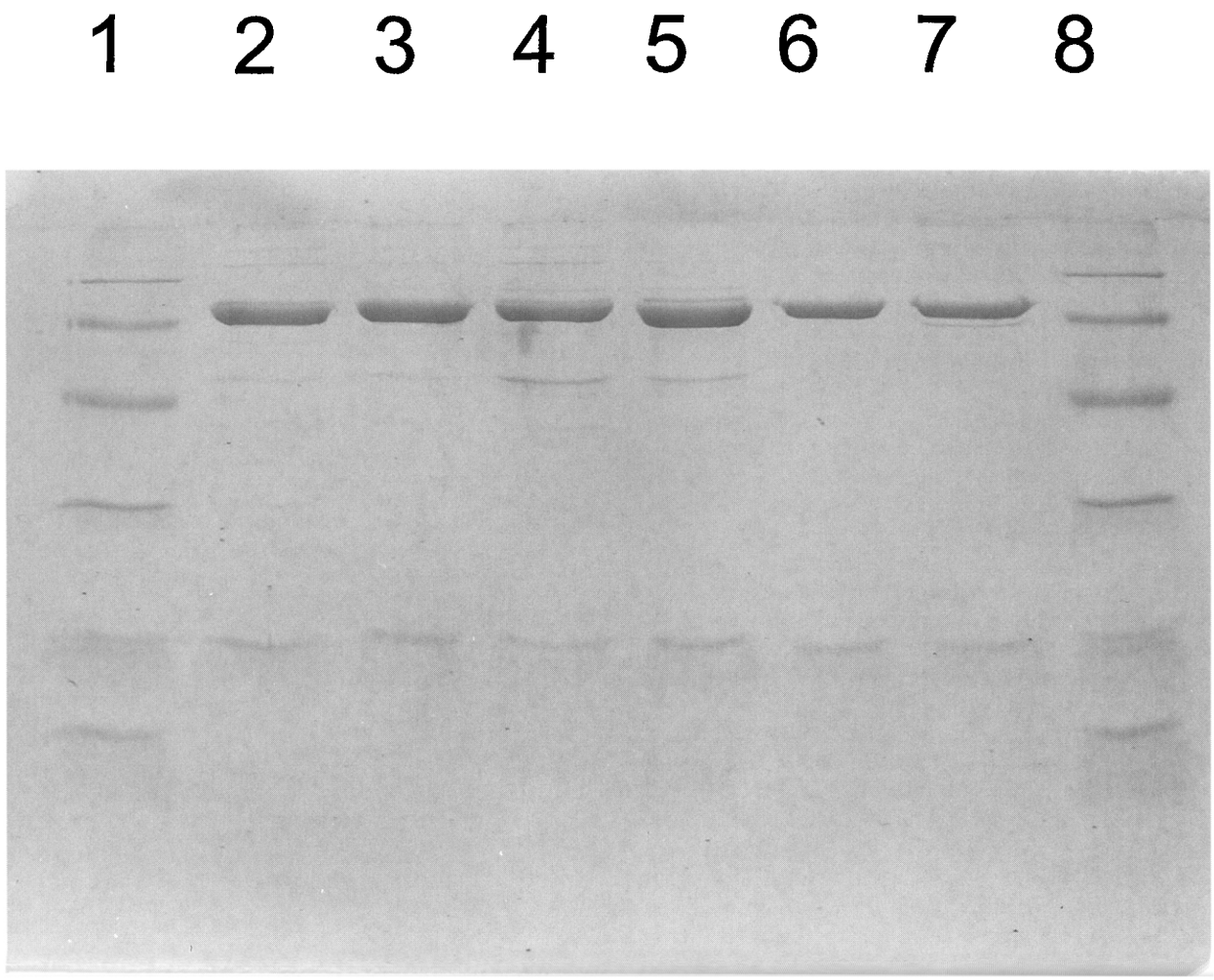

Fig. 1. 12.5% acrylamide SDS Tris-glycine gel, stained with Coomassie Brilliant Blue. The calpains shown are: wild-type, C105S, H262A, N286A, N286D and W288Y (lanes 2–7, left to right). Molecular weight standards (lanes 1 and 8) correspond to 97, 66, 45, 31, 21 and 14 kDa.

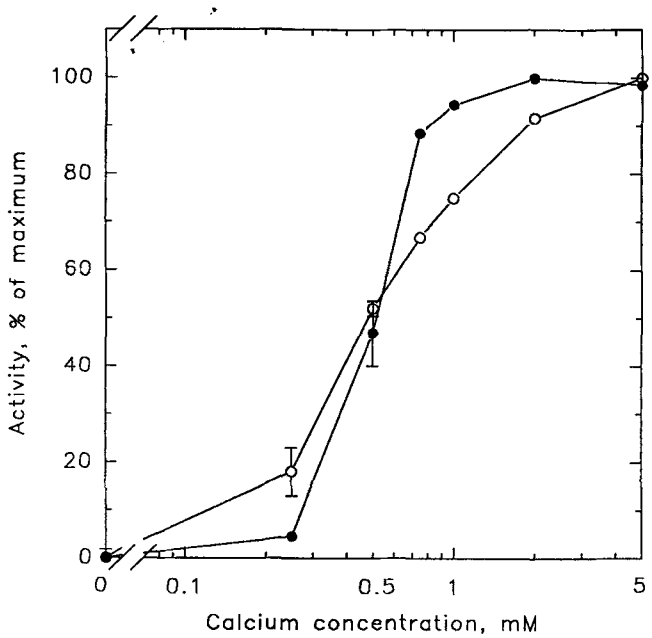

Fig. 2. Ca^{2+}-dependence of calpain activity. Enzyme samples were dialysed against 5 mM EDTA, and their activity in the standard casein assay was measured at net Ca^{2+} concentrations from 0.25–5 mM. Control values observed in the presence of 5 mM EDTA were set at 0%. The points represent the mean and standard deviations of triplicate observations. Wild-type calpain, ● W288Y, ○.

Aggregation of the mutant enzymes in the presence of 2 mM Ca^{2+} was measured by means of right-angled light scattering, and was used as an indication that the protein was folded sufficiently well to undergo a Ca^{2+}-induced conformational change. The mutants and wild-type calpains showed similar aggregation behaviour (Table 1), with the exception of W288Y, which aggregated more slowly.

4. Discussion

All of the mutants behaved indistinguishably from the wild-type enzyme during expression and purification, as dimers of 80- and 21-kDa subunits. Densitometry of Coomassie-stained gels of the purified mutants showed subunit ratios identical to that of the wild-type enzyme, consistent with a subunit stoichiometry of 1:1 (Fig. 1). Apart from W288Y which is discussed later, the responses of the mutants to Ca^{2+} were very similar to that of the wild-type enzyme. The available evidence of soluble expression, dimer formation, chromatographic behaviour and Ca^{2+}-dependent aggregation therefore suggests that the mutant proteins were correctly folded.

The active site of papain contains an S^-/ImH^+ ion pair (C25/H159), which is thought to be stabilised by a hydrogen bond between the side chains of H159 and N175 [6]. Since these residues are conserved in over 95% of all cysteine proteinases [5,9], it has been commonly assumed that the corresponding residues in calpain were C105, H262, and N286. The mutation C105S in calpain produced inactive enzyme, as observed previously in the C105A mutant [8]. The lack of activity of the calpain H262A mutant confirms the previous suggestion based on kinetic and pH data that a histidine was involved in the active site [10], and identifies the particular histidine residue. The lack of activity of the N286A mutant, in turn, suggests that a hydrogen bond between H262 and N286 is important for stabilisation of the ion pair in calpain. These results provide strong experimental evidence that cysteine-105, histidine-262 and asparagine-286 constitute the catalytic triad in calpain.

Conversion of the papain active site cysteine residue to serine (as with the calpain C105S mutant) caused loss of activity, which was attributed to a difference in length between a C-O and C-S bond [15,16]. This may not however be the only factor involved. The active site thiol group in the cysteine proteinases is ionized, and therefore strongly nucleophilic, because of its interaction with a histidine residue. When the cysteine is replaced with a serine residue, the histidine and asparagine residues in the mutant triad are apparently unable to promote sufficiently the nucleophilicity of the serine oxygen atom. Although some cysteine proteinases have been successfully converted to serine proteinases, these were viral 'trypsin-like' cysteine proteinases which may have an aspartic or glutamic acid residue, rather than an asparagine, in their catalytic triads [17,18]. Conversely, it is of interest to note that double mutation of trypsin to generate a cysteine/histidine/asparagine triad resulted in loss of activity [19].

The amide protons of N175 in papain are in a hydrophobic environment close to W177 [20]. Mutations of N175 in propapain have shown that it is required for normal processing and activity of the enzyme [21], and more recent work suggests that mutations in this position affect both structure and function (R. Menard, pers. commun.). In calpain, we thought that an aspartate at position 286 might increase the reactivity of C105 by interacting more strongly with H262, but in practice the N286D mutant had very low activity. It is not yet clear whether this is due to a failure of D286 to adopt a conformation capable of interacting with H262; or to a change in conformation of W288 caused by the hydrophilic nature of D286. Although this tryptophan is totally conserved in all calpain and papain-like sequences, its role is not clear, and several possibilities exist. It may form part of the S_1' binding site, (A. Storer, pers. commun.), it may shield the asparagine-histidine hydrogen bond from water [6], or it may interact directly with the ion pair histidine, as in the ribonuclease barnase [6,22]. The calpain W288Y mutant retained some activity (~5% of the wild-type) indicating that although not absolutely essential, this tryptophan residue must make a significant contribution either to catalysis or to substrate binding. The slightly altered response of the W288Y mutant to Ca^{2+} may imply some change in conformation.

Table 1
Calcium-dependent proteolysis and aggregation of calpain mutants

Calpain[a]	Specific activity[b]	Aggregation[c]
Wild-type	2,300	0.13
C105S	0	0.11
H262A	0	0.15
N286A	0	0.10
N286D	22	0.13
W288Y	128	0.06

[a]The wild-type or mutant large subunits were coexpressed with the 21-kDa small subunit and purified.
[b]Specific activity in the standard casein assay, U/mg protein.
[c]Arbitrary units, indicating the rate of increase of light scattering signal/s.

Other amino acid residues in the immediate vicinity of the catalytic triad in calpain must influence the reactivity of the central nucleophilic group, as well as contributing to substrate specificity. It is hoped that the recent development of an efficient expression system for calpain [8,12] will permit the combined use of mutagenesis and crystallization to investigate the fine details of its structure and mechanism.

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